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Title: **HIGHLY EFFICIENT MUTAGENESIS METHOD WITH THE USE OF PSORALEN DERIVATIVES**

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HIGHLY-EFFICIENT ~~MUTAGENESIS METHOD WITH~~ THE USE OF PSORALEN DERIVATIVES

Technical Field of the Invention

The present invention relates to a method for mutating a gene of a vertebrate animal, preferably zebrafish by using a psoralen derivative, preferably trimethylpsoralen; a method for preparing the gene mutated by the method; and a method for analyzing the function of the gene of the vertebrate animal by using the method.

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Background Art

In the last several years, a mass-scale mutant screening was conducted by using zebrafish, mainly in Germany and the U.S., so as to comprehensively isolate factors responsible for the vertebral development mechanism, and several hundreds of genes responsible for the development have been reported [Driever, W. et al., (1996) Development 123, 37-46.; Haffter, P. et al., (1996) Development 123, 1-36].

The mutagen used in the project (mutagenic substance for mutant] is N-ethyl-N-nitrosourea (ENU) which has an action to substitute one base composing a gene with another base. Hence, the mutagen has a drawback such that it is difficult to identify and clone an intended mutated gene, by using the slight mutation as a marker, despite the merit of the high efficiency [Postlethwait, J. H. and Talbot, W. S. (1997) Trends Genet., 13, 183-190].

Alternatively, the Hopkins' group developed an elegant insertional mutagenesis method comprising inserting a retroviral vector in a chromosome and

thereby disrupt a gene, as a strategy directed to gene cloning [Gaiano, N. et al., (1996) Nature 383, 829-832]. Because the sequence of the inserted vector was known, the chromosomal region around the vector could simultaneously be recovered, by using the sequence as the marker. However, the efficiency was at least several tens times lower than that of ENU, so the insertional mutagenesis method was not suitable for mutagenesis at a comprehensive scale [Postlethwait, J. H. and Talbot, W. S. (1997) Trends Genet., 13, 183-190].

One of other approaches is chromosomal deletion mutagenesis method. The deletion per se prepared by the approach advantageously serves as a marker for the cloning of a mutated gene. The deletion exerts its power by approaches, such as representational difference analysis (RDA) [Cimino, G. D., Gamper, H. B., Isaacs, S. T. and Hearst, J. E. (1985) Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. Annu. Rev. Biochem. 54, 1151-1193.]. It has been known insofar that irradiation of γ ray or X ray induces chromosomal deletion. As the deletion induced by the irradiation is so large, involving the damage of the chromosome, however, it is difficult to isolate a mutation at a level of single gene locus. Thus, those methods were not suitable for detailed research works of mutation, because the methods induced large deletion or disruption in the chromosome of zebrafish [Chackrabarti, S. et al., (1983) Genetics 103, 109-124.; Mullins, M.C. et al., (1994) Curr. Biol. 4, 189-202.].

Additionally, it is considered that the elucidation of the development mechanism by means of gene mutation possibly functions as a potent genetic approach, so as to isolate and identify the development factors and to additionally elucidate the development and function of each organ such as brain and the

functions of genes responsible therefor. Particularly, the systematic genetic analysis in vertebrate animals has become very important. Zebrafish has a great number of advantages among vertebrate animals, and it is observed that the rapid progress of the development of an organ such as brain in the growing transparent embryo [Stuemer, C. A. O. (1988) Retinotopic organization of the developing retinotectal projection in the zebrafish embryo. J. Neurosci. 8, 4513-4530].

It has additionally been known that trimethylpsoralen (4, 5', 8-trimethylpsoralen; abbreviated as TMP hereinbelow) known as one of DNA crosslinking agents highly frequently induces small chromosomal deletions in *Escherichia coli* and *Caenorhabditis elegans*.

Accordingly, the development of a new gene mutagenesis system enabling cloning at a high frequency in vertebrate animals, particularly zebrafish, has been desired.

The present inventors have counted the mutation frequency of a specific gene locus and have made a pilot screening. Consequently, the inventors have confirmed that TMP induces mutation even in vertebrate animals and that the TMP mutagenesis method is efficient. In order to confirm that the TMP mutagenesis method is practically functional, still furthermore, the inventors have analyzed mutants with specific abnormalities in the nervous system, as recovered by the TMP mutagenesis method. Based on these results, the inventors have found that the TMP mutagenesis method is an effective method, for isolating mutants of vertebrate animals, particularly zebrafish, and for analyzing the mutants at molecular level. Thus, the invention has been achieved.

Disclosure of the Invention

The invention relates to a method for mutating a gene of vertebrate animals, preferably zebrafish, by using a psoralen derivative, preferably trimethylpsoralen, and a method for preparing the gene mutated by the method.

Additionally, the invention relates to a method for analyzing the function of a gene of a vertebrate animal, comprising inducing mutagenesis in a gene region containing a pyrimidine base by using a psoralen derivative and expressing the mutated gene, and examining the correlation.

Brief Description of the Drawings

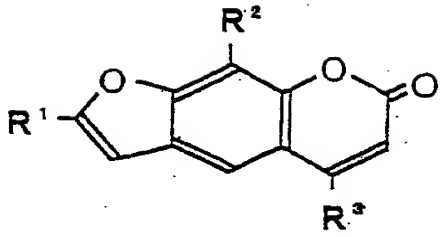
Fig. 1 depicts a schematic view of the TMP mutagenesis method;

Fig. 2 depicts the immunostaining of presumptive tectal neurons of the wild-type (A) and *ntn* mutant (B) embryos 38 hours after the development with anti-acetylated tublin antibody, in photographs of the side views; and

Fig. 3 depicts the trifacial node sensory neurons (A, B), Rohon-Beard sensory neurons (C, D) and posterior primary motor neurons (E, F) of the wild-type (A, C, E) and *edw* mutant (B, D, F) embryos 28 hours after the development, in photographs of the side views of the left head tops.

Best Mode for Carrying out the Invention

The psoralen derivative of the invention is satisfactorily psoralen per se or a derivative thereof, given that psoralen or the derivative thereof can functionally induce crosslinks in nucleic acids such as DNA by ultraviolet irradiation or X-ray irradiation, to delete a region including the crosslinked site. Preferably, the psoralen derivative of the invention is a compound represented by the following formula I:



(I)

wherein R^1, R^2 and R^3 independently represent hydrogen atom or a hydrocarbon group.

The hydrocarbon group in the general formula I includes for example a linear or branched alkyl group with 1 to 30 carbon atoms, preferably 1 to 20 carbon atoms, more preferably 1 to 10 carbon atoms, which is more preferably a lower alkyl group; a linear or branched alkenyl group with 2 to 30 carbon atoms, preferably 2 to 20 carbon atoms, more preferably 2 to 10 carbon atoms; a monocyclic, polycyclic or condensed cyclic cycloalkyl group with 5 to 30 carbon atoms, preferably 5 to 20 carbon atoms, more preferably 6 to 10 carbon atoms; and a monocyclic, polycyclic or condensed cyclic aryl group with 6 to 30 carbon atoms, preferably 6 to 20 carbon atoms, more preferably 6 to 10 carbon atoms.

Additionally, the alkyl group, alkenyl group, cycloalkyl group and aryl group in the general formula I may further have substituents; or these groups may be substituted with each other. For example, alkyl-substituted cycloalkyl group, alkyl-substituted aryl group, aryl-substituted alkyl group (aralkyl group), and cycloalkylalkyl group are listed.

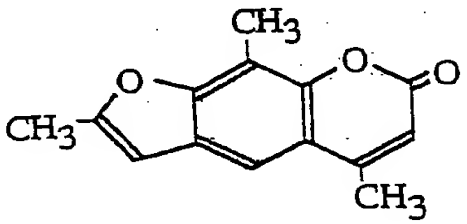
Other substituents include for example alkoxyl group, alkylthio group, dialkylamino group, and trialkylsilyl group, all these groups comprising the alkyl group described above; halogen atoms such as chloride, bromide and fluoride; alkylenedioxy groups such as methylenedioxy group and 2,2-

dimethylmethylenedioxy group; and cyano group.

The substituents are preferably lower alkyl groups such as methyl group, ethyl group, n-propyl group, isopropyl group, and t-butyl group; aryl groups such as phenyl group and naphthyl group; lower alkoxyl groups such as methoxy group, ethoxy group and n-propoxy group; di(lower alkylamino) groups such as dimethylamino group, diethylamino group, and dipropylamino group; lower alkyl-substituted silyl groups such as trimethylsilyl group, triethylsilyl group, dimethylethylsilyl group and dimethyl(t-butyl)silyl group; halogen atoms such as chloride and fluoride; alkylenedioxy groups such as methylenedioxy group and 2,2-dimethylmethylenedixoy group; and cyano group.

Specific examples of R^1, R^2 and R^3 in the general formula I include for example lower alkyl groups such as methyl group, ethyl group, n-propyl group, isopropyl group, n-butyl group, t-butyl group and hexyl group; lower alkenyl groups such as vinyl group, propenyl group and butenyl group; cycloalkyl groups such as cyclohexyl group and cyclopentyl group; aryl groups such as phenyl group and naphthyl group; aralkyl groups such as benzyl group and phenethyl group.

The psoralen derivative of the invention is preferably trimethylpsoralen (TMP) represented by the following formula II:



(II)

Under ultraviolet irradiation, TMP (4-5′-8-trimethylpsoralen) forms a covalent bond with a pyrimidine base composing a DNA double helix, to make an

interstrand crosslink in the DNA double strand. A region around the crosslinked site is excised through the DNA recombinational repair mechanism essential to biological organisms, so that a small deletion occurs [Cimino, G. D., et al., (1985) Annu. Rev. Biochem., 54, 1151-1193.].

In the case where an essential gene is present in the deleted region, the function thereof is lost, involving the emergence of a mutant. Because such a marker as the deletion at the chromosomal level is added, unlike the ENU point mutation, the mutated gene can be identified and cloned, by cloning a chromosomal fragment around the mutated site by a specific method, for example the RDA method (Representational Difference Analysis) [Lisitsyn, N. et al., (1993), Science, 259, 946-951.] and subjecting the chromosomal fragment to chromosomal walking, so as to determine the deleted site.

The results of the pilot screening conducted by the inventors suggested that the mutant isolation frequency by the gene mutagenesis method using psoralen derivatives, particularly TMP, was at the same level as that of ENU.

The gene mutagenesis method using TMP for *Caenorhabditis elegans* was reported previously [Yandell, M. D. et al., (1994) Proc. Natl. Acad. Sci. USA, 91, 1381-1385.]. Nevertheless, the present method comprising treating a vertebral gene, particularly vertebral sperm with TMP and inducing mutagenesis in the embryo by artificial fertilization is a novel methodology with no such example found in the past. The method is quite unique in that mutant strains of zebrafish can totally be isolated and that the cloning of these mutated genes enables the molecular level analysis of all the vertebral functions.

In order to develop an efficient deletion mutagenesis method in zebrafish, the inventors have carried out experiments with TMP as one of DNA crosslinking

reagents [Driever, W., Solnica-Krezel, L., Schier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J. and Boggs, C. (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123, 37-46.]. Fig. 1 depicts the summary of the experimental method. Fig. 1 is now described briefly. Sperm collected from males of the zebrafish AB strain was placed in a TMP solution, which was then subjected to ultraviolet irradiation. The sperm after the treatment was artificially fertilized with a wild-type egg, which was then grown into an F1 individual with the mutagenized genome in hetero (+/m). The F1 individual was mated with the wild-type AB strain (+/+); and one half thereof generated an F2 population with a specific mutated gene locus in hetero. One pair per 4 pairs of the F2 siblings (+/+ or +/m) mated together generated an F3 embryo (white embryo) having mutation in a homozygous fashion (m/m) and expressing the phenotype of the mutant, at a ratio of 1/4.

Zebrafish males of the AB strain were anesthetized with 0.03 % (W/V) 3-aminobenzoate ethyl ester and then inserted at their spine positions between the slits of a sponge block. From the 3 to 5 males was aspirated sperm with a 20-μl capillary tube, which was then suspended in 100 μl of Hank's sodium chloride solution containing 3 ng/ml to 300 ng/ml TMP and 1 % dimethyl sulfoxide (DMSO). After the resulting suspension was left to stand on ice for 5 minutes, 10-μl portions of the suspension were dropwise added to and arranged on a 2-mm thick plastic petri dish; the petri dish was then placed on the ultraviolet irradiator of a DNA crosslinking apparatus (TFL-20M, manufactured by Vilber Lourmat, Co. Ltd., France); through the bottom of the petri dish, the sperm was irradiated with ultraviolet light under conditions of 312-nm wavelength and 0.02 J/cm^2.

The mutagenized sperm was artificially fertilized with a fresh normal egg squeezed out of the abdomen of AB-strain females under anesthesia, while the abdomen was gently pressed, according to a routine method (day 0) [Stuemer, C. A. O. (1988) Retinotopic organization of the developing retinotectal projection in the zebrafish embryo. J. Neurosci. 8, 4513-4530.]. The fertilized egg was placed at 28 °C in darkness for 12 hours, so as to grow an F1 individual according to a routine method [Allende, M. L., Amsterdam, A., Becker, T., Kawakami, K., Gaiano, N. and Hopkins, N., (1996) Insertional mutagenesis in zebrafish identifies two novel genes, pescadillo and dead eye, essential for embryonic development. Gene Dev. 10, 3141-3155.].

Almost all of the embryos treated under the same conditions except for no TMP addition were grown to mature fish individuals (580 out of 594). Among the embryos treated with 30 ng/ml TMP, only 57 % (371 out of 650) could survive until day 9; among the embryos treated with 300 ng/ml TMP, only 9.5 % (49 out of 515) could survive until day 9.

Among the embryos developed from the sperm treated with 30 ng/ml TMP, 70 % or more (122 out of 170) exhibited various abnormalities until day 2 postfertilization.

Meanwhile, the number of abnormal individuals under conditions without both or either one of TMP and ultraviolet light was very small (2 % or less).

In order to examine the efficiency of the TMP mutagenesis method on the basis of the assay of the mutation frequency of a specific gene locus, subsequently, experiments of tester mutation were carried out by using zebrafish with a spotted pigmentation. The inherited character of the spotted pigmentation used in the tester mutation was recessive and transmitted in the genetic mode according to the

Mendel's Law, indicating that the character was derived from a single gene locus. The inventors treated the sperm of AB-strain males separately with 30 ng/ml and 300 ng/ml TMP, for artificial fertilization with the egg of tester-strain females. Thirty days later, 6 out of 1181 F1 individuals derived from the 30 ng/ml TMP treatment and 3 out of 130 F1 individuals derived from the 300 ng/ml TMP treatment exerted pigment mutation in dotted pattern. Additionally, no individual with pigment mutation was observed among 866 F1 individuals treated only with ultraviolet irradiation. Consequently, the mutation frequencies of 30 ng/ml TMP and 300 ng/ml TMP were calculated as 0.5 % and 2 %, respectively, which demonstrates that the TMP mutagenesis method is extremely efficient in zebrafish.

During a term from day 1 to day 5 postfertilization, screening (F2 screen; see Fig. 1) was performed on the basis of the morphology of F3 embryo under observation with a stereomicroscope and the response to contact stimulus. Then, mutants of 10 strains were isolated mainly from 26 F1 individuals derived from the sperm treated with 30 ng/ml TMP. Furthermore, 2 strains were also isolated from the screen of the haploids generated in a parthenogenesis manner via the activation of the F1 egg with functionally impotent sperm (Stuemer, C.A.O. (1988) Retinotopic organization of the developing retinotectal projection in the zebra fish embryo. J. Neurosci. 8, 4513-4530.). The resulting mutant strains are as follows: growth degeneracy (4 strains), small head and small eyes (3 strains), no motility (2 strains), edema accompanied by ventricular enlargement (one strain), short length (one strain) and tectal necrosis (one strain). All mutants were recessive and lethal in the genetic mode according to the Mendel's Law.

In order to confirm that the TMP mutagenesis method was sufficiently

functional, the inventors analyzed 2 mutant strains with abnormalities in the nerve system in more detail. In the case of the embryo of a mutant (*ntn)* with no tectal neuron, opaqueness appeared selectively in the tectum and eye in approximately around 40 hours. When stained with the anti-acetylated tublin monoclonal antibody (Sigma, Co.) [Haffter, P., Granato, M., Brand, M., Mullins, M. C., Hammerschmidt, M., Kane, D. A., Odenthal, J., van Eeden, F. J. M., Jiang, Y.-J., Heisenberg, C.-P., Kelsh, R. N., Furutani-Seiki, M., Vogelsang, E., Beuchle, D., Schach, U., Fabian, C., and Nuesslein-Volhard, C. (1996) The identification of genes with unique and essential functions in the development of the zebrafish, Danio rerio. Development 123, 1-36.], the axonogenesis of the presumptive tectal neuron was prominently progressed in the wild-type embryo within 37 to 39 hours, to form a tectal neuropil (Fig. 2A). Almost no development of any presumptive tectal neuron occurred in the embryo of the no tectal neuron mutant (*ntn*) at the same period of time (Fig. 2B), although no difference in staining was observed in the remaining regions of the embryo. The results of these analyses indicate that the *ntn* mutation affects the development of tectal neuron and eye. In terms of the invasion of tectum and eye, the *ntn* mutant is common to several previously reported mutants (Class I and Class III) with abnormality in the neural development [Chen, C. and Tonegawa, S. (1997) Molecular genetic analysis of synaptic plasticity, activity-dependent neural development, learning and memory in the mammalian brain. Annu. Rev. Neurosci. 20, 157-184, Chackrabarti, S., Streisinger, G., Singer, F. and Walker, C. (1983) Frequency of γ-ray induced specific locus and recessive lethal mutations in mature germ cells of the zebrafish, Brachydanio rerio. Genetics 103, 109-124.].

Fig. 2 depicts the immunostaining with anti-acetylated tublin antibody of

the presumptive tectal neurons in the embryos of wild type (A) and no tectal neuron (*ntn*) (B) in 38 hours after the development, as shown in color photographs of the side views. The left head tops. The arrow indicates TPC (the tracts of posterior commissures). "T" represents presumptive tectum. The scale bar in Fig. 2 corresponds to 100 μm.

Furthermore, the inventors have found an *edawakare* mutant (*edw*) as a mutant with small head and no body motility. Abnormalities in the sensory neuron and muscle were first found in the mutant, which corresponds to the characteristic property that the mutant never responded to gentle contact to its cauda, around 24 hours later. In 20 hours after the development, the peripheral axons of the trifacial node and Rohon-Beard sensory neurons were abnormally extensive in the *edw* mutant. In the wild type, meanwhile, the peripheral axon of the trifacial nodal cell projected in the epidermis of the posterior head and the anterior egg yolk cavity (Fig. 3A). In the wild type, however, the axon projected in a more diverse manner and reached the anterior head. Additionally, the axon was thin (Fig. 3B).

In 28 hours after the development, the peripheral axon of the wild-type Rohon-Beard neuron was elongated approximately toward the ventral and caudal directions all over the body (Fig. 3C). In the mutant, alternatively, the axon was actively branched and elongated toward various directions (Fig. 3D). In 20 hours after the development, the muscle fiber under way of formation in the wild type was partially striped in pattern. The muscle fiber in the mutant was never striped. No change was observed in the *edw* mutant in 36 hours; however, in the wild type, the skeletal muscle formed a sharp striped structure by that time.

Still additionally, the inventors have found in 28 hours after the

development that the posterior primary motor neuron (CaP) of the mutant was abnormal. In the wild type, the CaP axons were elongated to the ventral myotome (Fig. 3E), while in the *edw* mutant, the elongation of almost all of the CaP axons of the anterior 15 myotomes stopped intermediately in the myotomes (Fig. 3F).

Fig. 3 depicts the trifacial node sensory neurons (A and B), Rohon-Beard sensory neurons (C and D) and posterior primary motor neurons (E and F) in the embryos of wild types (A, C and E) and *edw* (B, D and F), 28 hours after the development, in color photographs of the side views of the left head tops. The arrow heads in Fig. 3 represent the activated sensory axons (B and D) and the degenerated motor axon (F) in the *edw* embryo. The scale bars in Fig. 3 correspond 100 μm (A and E) or 50 μm (C).

In all the *edw* mutants analyzed, all these characters herein referred to were observed with reproducibility. These results indicate that the *edw* mutation affects not only the projection of trifacial node and Rohon-Beard sensory neurons but also the formation of body wall muscle fiber.

Alternatively, the degeneracy of the CaP axon is possibly ascribed to the abnormality of body wall muscle. Up to now, several types of mutants (*fub, fro, slo*) without any motility or formation of muscular striped structure have been reported [Gaiano, N., Amsterdam, A., Kawakami, K., Allende, M., Becker, T. and Hopkins, N. (1996) Insertional mutagenesis and rapid cloning of essential genes in zebrafish. Nature (London) 383, 829-832, Sladek, F. M., Melian, A. and Howard-Flanders, P. (1989) Incision by UvrABC excinuclease is a step in the path to mutagenesis by psoralen crosslinks in Escherichia coli. Proc. Natl. Acad. Sci. USA 86, 3982-3986.]. No report has yet been published about the abnormality in

sensory neuron.

The inventors have developed a methodology of highly efficient mutagenesis method of zebrafish as one of vertebrate animals, using the DNA crosslinking reagent psoralen derivative. The mutation frequency of a specific gene locus and the high efficiency of the mutagenesis method with the psoralen derivative as shown in the pilot screening possibly enable the isolation of a great number of zebrafish mutants even at a small scale.

The concentration of the psoralen derivative of 30 ng/ml, is probably too high. The reason is that the majority of the F1 embryos at the TMP experiments were abnormal because of the presence of possible dominant lethal mutation therein. Actually, even the psoralen derivative at a concentration of 3 ng/ml could efficiently be used for mutant isolation.

The concentration of the psoralen derivative, preferably TMP for use in the method of the invention is 0.01 to 300 ng/ml, preferably 0.1 to 100 ng/ml, more preferably 0.1 to 50 ng/ml, and still more preferably 1 to 30 ng/ml.

As to the method for prescribing the psoralen derivative in accordance with the method of the invention, the psoralen derivative is used for the treatment of sperm as described above but is also dosed by other methods.

The method of the invention comprises treating a test subject with the psoralen derivative and subsequently irradiating high-energy beams such as ultraviolet light, X ray and γ ray on the test subject. One of these high-energy beams can appropriately be selected, depending on the type of the test subject, and the type and quantity of the psoralen derivative for use in such treatment.

The vertebrate animal in accordance with the invention includes vertebrate animals excluding humans, for example fish and mammals.

The most significant characteristic aspect of the mutagenesis method of the invention is method resides in the characteristic action of the psoralen derivative, particularly TMP. The activation of TMP and the like under ultraviolet irradiation induces the emergence of a covalent bond with a pyrimidine base in a DNA double helix structure, to form crosslinks in the DNA [Driever, W., Solnica-Krezel, L., Shier, A. F., Neuhauss, S. C. F., Malicki, J., Stemple, D. L., Stainier, D. Y. R., Zwartkruis, F., Abdelilah, S., Rangini, Z., Belak, J. and Boggs, C. (1996) A genetic screen for mutations affecting embryogenesis in zebrafish. Development 123, 37-46.]. It is known in *Escherichia coli* and *Caenorhabditis elegans* that the excision of a base in a crosslinked interstrand site of the DNA double strand and the recombinational repair often cause a small deletion in the genome DNA [Chitnis, A. B. and Kuwada, J. Y. (1990) Axonogenesis in the brain of zebrafish embryos, J. Neurosci. 10, 1892-1905, Furutani-Seiki, M., Jiang, Y.-J., Brand, M., Heisenberg, C.-P., Houart, C., Beuchle, D., van Eeden, F. J. M., Granato, M., Haffter, P., Hammerschmidt, M., Kane, D. A., Kelsh, R. N., Mullins, M. C., Odenthal, J. and Nuesslein-Volhard, C., (1996) Neural degeneration mutants in the zebrafish, Danio rerio. Development 123, 229-239.]. Such deletion serves as an indicator essential for the cloning of mutated genes on application of a suitable method.

We have actually carried out RDA (Representational Difference Analysis) [Cimino, G. D., Gamper, H. B., Isaacs, S. T. and Hearst, J. E. (1985) Psoralens as photoactive probes of nucleic acid structure and function: organic chemistry, photochemistry, and biochemistry. Annu. Rev. Biochem. 54, 1151-1193.] between sibling mutants and normal zebrafish individuals, so that the DNA fragment deleted in the genome of the *edw* mutant could be cloned.

Thus, the mutagenesis method of the invention works as a valuable approach, other than the ENU mutagenesis method [Russell, W. L., Kelly, E. M., Hunsicker, P. R., Bangham, J. W., Maddux, S. C., and Phipps, E. L. (1979) Specific-locus test shows ethylnitrosourea to be the most potent mutagen in the mouse. Proc. Natl. Acad. Sci. USA 76, 5818-5819, Wilson, S. W., Ross, L. S., Parrett, T. and Easter, S. S. Jr. (1990) The development of a simple scaffold of axon tracts in the brain of the embryonic zebrafish, Brachydanio rerio. Development 108, 121-145.] and the insertional mutagenesis method [Mullins, M. C., Hammerschmidt, M., Haffter, P. and Nuesslein-Volhard, C. (1994) Large-scale mutagenesis in the zebrafish: in search of genes controlling development in a vertebrate. Curr. Biol. 4, 189-202.].

Examples

The present invention will now be described in detail by referring to examples, but the invention is not limited to these specific examples.

Example 1

Zebrafish males of the AB strain were anesthetized with 0.03 % (W/V) 3-aminobenzoate ethyl ester and then inserted at their spine positions between the slits of a sponge block. Sperm was aspirated from the 3 to 5 males with a 20-μl capillary tube, which was then suspended in 100 μl of Hank's sodium chloride solution containing 3 ng/ml to 300 ng/ml TMP and 1 % dimethyl sulfoxide (DMSO).

After the resulting suspension was left to stand on ice for 5 minutes, 10-μl portions of the suspension were dropwise added to and arranged on a 2-mm thick plastic petri dish; the petri dish was then placed on the ultraviolet irradiator of a

DNA crosslinking apparatus (TFL-20M, manufactured by Vilber Lourmat Co., Ltd., France), through the bottom of the petri dish, sperm was irradiated with ultraviolet light under conditions of 312-nm wavelength and 0.02 J/cm^2.

The mutagenized sperm was artificially fertilized with a fresh normal egg squeezed out of the abdomen of AB-strain females under anesthesia, while the abdomen was gently pressed, according to a routine method (day 0). The fertilized egg was placed at 28 °C in darkness for 12 hours, so as to grow an F1 individual according to a routine method.

Among the embryos treated with 30 ng/ml TMP, only 57 % (371 out of 650) could survive until day 9; among the embryos treated with 300 ng/ml TMP, only 9.5 % (49 out of 515) could survive until day 9.

Among the embryos treated with 30 ng/ml TMP, 70 % or more (122 out of 170) exerted various abnormalities until day 2 postfertilization.

Comparative Example 1

The same treatment as in Example 1 was carried out under the same conditions, except for no addition of TMP. Consequently, almost all of the embryos were grown into the adult fish (580 out of 594).

Alternatively, the number of abnormal individuals under conditions without both or either one of TMP and ultraviolet light was very small (2 % or less).

Example 2

Using zebrafish in a dotted pattern, experiments of tester mutation were carried out with the same treatment as in Example 1.

Male sperm of the AB strain was treated with TMP at 30 ng/ml and 300

ng/ml, respectively, which was then artificially fertilized with the egg of a tester-strain female. Thirty days later, 6 out of 1181 F1 individuals derived from the 30 ng/ml TMP treatment and 3 out of 130 F1 individuals derived from the 300 ng/ml TMP treatment exerted pigment mutation in dotted pattern.

Additionally, no individual with pigment mutation was observed among 866 F1 individuals treated only with ultraviolet irradiation. Consequently, the mutation frequencies of 30 ng/ml TMP and 300 ng/ml TMP were calculated as 0.5 % and 2 %, respectively, which demonstrates that the TMP mutagenesis method is extremely efficient in zebrafish.

During a term from day 1 to day 5 postfertilization, screening (F2 screen) was performed on the basis of the morphology of F3 embryo under observation with a stereomicroscope and the response to contact stimulus. Then, mutants of 10 strains were isolated mainly from 26 F1 individuals derived from the sperm treated with 30 ng/ml TMP.

Furthermore, 2 strains were also isolated from the screen of the haploids generated in a parthenogenesis manner via the activation of the F1 egg with functionally impotent sperm. The resulting mutant strains are as follows: growth degeneracy (4 strains), small head and small eyes (3 strains), no motility (2 strains), edema accompanied by ventricular enlargement (1 strain), short length (1 strain) and tectal necrosis (1 strain). All mutants were recessive and lethal in the genetic mode according to the Mendel's Law.

In order to verify the sufficient functionality of the TMP mutagenesis method, the inventors analyzed 2 mutant strains with abnormalities in the nerve system in more detail. The results of *ntn* mutant are shown in the color photographs of Fig. 2.

As another mutant, an *edawakare* mutant (*edw*) with small head and no body motility was found. Abnormalities in the sensory neuron and muscle were first found in the mutant, which corresponds to the characteristic property that the mutant never responded to gentle contact to its cauda around 24 hours later. The color photographs of Fig. 3 show the peripheral axons of the trifacial node and Rohon-Beard sensory neurons and the CaP axons in wild type and the *edw* mutant with abnormalities observed in their extension.

Industrial applicability

The invention provides a highly efficient preparation system of a mutant of vertebrate animals, particularly zebrafish; the invention also provides a method characterized in that a mutated gene can be cloned by using a deleted part as a marker on comparison with the normal chromosome because a small deletion is induced in the chromosome and that a great number of mutants with a cloning possibility can be prepared even at a small scale.